e-ISSN No.: 2582-4228

Journal of Indian Association for Environmental Management

Journal homepage: www.http://op.niscair.res.in/index/php/JIAEM/index

Development in Bacteriology and Bacteria Detection Techniques with Special Reference to Marine Bacteria

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Submitted: 22 August 2025 Revised: 13 November 2025 Accepted: 14 November 2025

***Abstract*: The review article is about overview the different groups of bacteria those are present in nature and perform important physiological and biochemical functions as survival strategy and functional diversity upon energy substrate. As bacterial families depending on nutrients using cultural methods for growth and division, biochemical characterizations have been used for identification of bacteria are described in earlier editions of Bergey's manual of determinative bacteriology. Further certain bacteria as phototrophic, heterotrophic, and pathogens have distinguished functional roles in habitats, recycling and diseases are described based on group identification are described in this review in table form. Biochemical tests are applied to bacterial culture to know the characteristics of species. Nevertheless now days genetic base 16S RNA, DNA base sequences characterization using polymerase chain reactions, G + C base ratio, and other recent advanced methods and technologies are used in phylogenetic classification which quickly provide results to the researchers. Genetic sequences is directed to transcribe RNA to code and translate to amino-acids and produced small amount of proteins in all bacteria, living organisms and specific Infectious factor (toxin) in pathogens. Moreover identification needs advanced methods and techniques for quantitative and qualitative determination of bacteria have to be conducted from field samples which are viable but nonculturable bacteria (VBNC) using Microscopic observations and Image-analysis, Epifluorimetry-Image analysis, Gene probes Polymerase chain reaction, Monoclonal antibodies, Biosensors and Laser cytometry.**

Keywords: Bergey's Manual, Bacterial Classification, Bacteria Detection Techniques, Habitat.

I. Introduction

The review research paper provides the development in bacteriology beginning of 20th when the development took place in the field of Microbiology with finding microscopic microorganisms vis bacteria were observed from the different habitats. These were observed in carbon source substrates belongs to Water, Soil and human environment. The researches were initiated and carried forward in Europe and U.S.A. The identification, characterization of bacteria using microscopic techniques i.e. Photomicrographs of higher resolution using compound microscope, confocal laser scanning microscopy (CLSM), EM techniques such as Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy SEM and coined recent advance molecular biology of bacteria and other microorganisms. The review research paper describes the bacteriological techniques as mention in abstract during the process up to recent development and useful for researcher. The paper is subsection in to taxonomic history, and modern techniques.

Taxonomic History

The Microorganisms have shown ubiquitous presence in the habitat using nutrients form soil, water, air and symbiotic associations in environment with plants and animals. The most complete and authoritative description of archaea, proteobacteria and eubacteria were compiled in Bergey's manuals. In spite of diversity of prokaryotes, these manual provides description of prokaryotic ecology, taxonomy, physiology, and systematic phylogeny.

David Hendrick Bergey trust had first published the Bergey's manual of determinative bacteriology in simplified

way. The nine editions of Bergey's manual of determinative bacteriology were published by Roberts Breed, E.G.D. Murray, Nathan R. Smith, and other authors in the years 1923, 1925, 1930, 1934, 1939, 1948, 1957, 1974 and 1994. Last two editions edited by different authors; Details of few bacterial families were given in table-1. Since that time, numerous unknown bacteria have been identified and classified **in** the manual by Roberts Breed, E.G.D. Murray, Nathan R. Smith, (1957), and the manual by Buchanan, R.E. & Gibbons, N.R, eds., (1974).

Bergey's Manual of determinative bacteriology, bacteria groups were organized based on phenotypic characteristics had published in nine editions between 1923 and 1994. Later on, with progressive attempt to sort out based on biochemical characteristics in the 4-volumes in Bergey's manual of systematic bacteriology, 1st edition, Williams and Wilkins, Baltimore, were published from 1984 through 1994 (Buchanan, R.E. & Gibbons, N.R, and Holt, John G., eds. 1994);.. This manual using every characterizing aspect of bacterial species and included information on all types of Gram-negative bacteria that were of medical and industrial importance were published 1884-1991. The following features of bacteria: energy and carbon source, mode of locomotion, morphology and Gram staining reaction, gaseous requirement and endospore formation ability were studied in Bergey's manual of systematic bacteriology published four volumes, (published by William and Wilkins, Baltimore Company of USA).

(I) Vol. I, it include Gram negative bacteria (1984).
(II) Vol. II, part A and B, it include the Proteobacteria and the Gamma Proteobacteria-Gram negative bacteria (1986).
(III) Vol. III, it includes bacteria with unusual cell wall like Archaebacteria, the Firmicutes & Gram positive bacteria (1989).
(IV) Vol. IV, it includes Actinomycetes and other Filamentous bacteria (1991).

The organization of Bergey's manual of systematic bacteriology were found impractical for identification place of several unknown bacteria (viable not culturable bacteria-VNCB) belong major taxa. However, it contains more checklists on the families, genera and species and is more up to date, later on introduction of molecular techniques have made identification possible of such bacteria e.g. 16rRNA sequences array, metagenomics, fatty acid profiles in bacterial species were arranged according to known phylogenetic relationships. The further 2nd editions Bergey's manual of systematic bacteriology, second edition bacteriology manuals were published in five volumes through 2001 to 2012 (https://.en.m.wikipedia.org. >wiki). Group of all the clinically important prokaryotes together as the first edition instead of pathogenic spp. were placed as on phylogenetic tree and thus scattered throughout the following five volumes.

- Bergey's manual of systematic bacteriology, 2nd ed., 2001, Vol. 1, The Archaea and the deeply branching and Phototrophic bacteria Springer-Verlag New York, NY.
- Bergey's manual of systematic bacteriology 2nd ed., 2005, Vol. 2, The Proteobacteria divided into three books- 2A: Introductory Essays; 2B: The Gamma Proteobacteria; 2C: Other classes of Proteobacteria parts A, B and C, Springer-Verlag New York, NY.
- Bergey's manual of systematic bacteriology, 2nd ed., 2009, Vol. 3: The low G+C, Gram negative bacteria, Springer-Verlag, New York, NY.
- Bergey's manual of systematic bacteriology, 2st ed., 2010, Vol. 4, The high G+C, Gram positive bacteria, Springer-Verlag, New York, NY.
- Bergey's manual of systematic bacteriology, 2nd ed., 2012, Vol. 5, The Bacteriodes, (obligate anaerobic bacteria-nonendospore-forming) Spirochetes, Fibro bacteria, Fusobacteria and Planktomycetes. Springer-Verlag New York, NY.

In these manuals, much work has done on sequencing of rRNA, DNA and proteins that have made the phylogenetic analysis of prokaryote feasible. It has more ecological information about individual taxa.

In Larger quantities, microorganisms have observed and recognized in formation of pedagogical strata and perform role in nitrogen cycle, mineral transformation i.e. phosphorus, sulfur, iron and other elements and rhizosphere associations of plants.

i. Census wise they may number billions g^{-1} of soil
ii. There are more than 400 named genera and an estimated 10^4 species
iii. Many of rDNA sequence patterns found in soil microorganisms are dissimilar to those of named bacteria
iv. Taxa containing noteworthy soil and water dwelling organisms relying heavily on rRNA sequence patterns subdivided the bacteria into the 12 phyla

Bacterial habitats

Thus, bacterial cells are distributed in various soil horizons and play important functional role in carbon cycle i.e. decomposition of organic matter, cellulose, hemi cellulose, lignin, other polysaccharides and transformation of hydrocarbon in soil. From different soil types have shown colonies on agar ((bacteria survival and utilization of different energy sources, (Alexander, 1977). Groups were identified, classified and quantified as

- 5 to 60 percent are of *Arthrobacter* sp.
- 7 to 67 percent of *Bacillus* sp.
- 3 to 15 percent of *Pseudomonas* sp.
- Up to 20 percent of *Agro bacterium* sp.
- 2 to 12 percent of *Alcaligenes* sp.
- 2 to 10 percent of *Flavobacterium* sp.
- < 5 percent of the colonies are derived from cells of *Corynebacterium* sp., *Micrococcus* sp., *Staphylococcus* sp., *Xanthomonas* sp., *Mycobacterium* sp., *Sarcina* sp.

As a result of human interventions and consequence, 21 bacterial genera *Acinetobacter* sp., *Agrobacterium* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Bacillus* sp., *Brevibacterium* sp., *Caulobacter* sp., *Cellulomonas* sp., *Clostridium* sp., *Corynebacterium* sp., *Flavobacterium* sp., *Hyphomicrobium* sp., *Metallogenium* sp., *Micrococcus* sp., *Mycobacterium* sp., *Pedomicrobium* sp., *Pseudomonas* sp., *Sarcina* sp., *Staphylococcus* sp., *Streptococcus* sp., and *Xanthomonas* sp.

- Phylogenetic distribution of eukaryote found on decaying logs and forest stuffs in forest are described relation with other groups to complete demarcation among groups. Eukaryota-Protozoa-ciliate, zoo-flagellates, amoebae, slime molds: chymists-oomycetes, dictyostelids (cellular slime mold): *Dictyostelium discoideum* and *Fuligo septico*, protostelids: *Ceratiomyxa fruticulosa*, and myxogastria: (plasmodial slime mold), algae mycota - zygomycetes, dikaryomycetes (Lecture notes - Microbiologist-2 with fish and aquatic environment.docx).
- Animalia-nematodes, mites, millipedes, centipedes, annelid worms, collembolans winged insects, (https://bio.libretexts.org>Botany2.04_protists>2).

Many bacteria could not be identified and exist in (viable but non-culturable bacteria (VBNC) are attempted to identified from different niches of the environment (Sagar, 2022).

In this article bio-geological origin for instance, certain bacterial families and bacterial groups were described based on Woese (1987) classified bacteria as: purple bacteria, green sulfur bacteria, Actinomycetes, Sporogenic bacilli, Cyanobacteria and Archaea- Euryarcheota-extreme halophiles, Methanogens; Crenarcheota, Thermo-acidophilic Crenarcheotes. For instance, Archaea are most common or dominant in habitats Extreme with respect to heat, osmotic stress, reducing capacity and limitation of resources to mineral substrates. Archaea characterized as dividing in to three main ecological groups

1. Thermophiles
2. Halophiles
3. Methanogens

Most of the prokaryotes, Archaea are thermophiles growing in hot springs communities (<80°C to >100°C) or halophiles are salt loving bacteria, growing in high salt environment and methanogens utilize organic substrates, growing in anaerobic environment indicated that their diversity is much more complex than earlier thought by Ward and Castenholz in bacterial phyla and their subdivision, (table-2).

- Methanogen are using carbon dioxide from acetate, format substrates in the habitats like which are lacking sources of less strongly bound oxygen sulfate and nitrate.
- Thus methanogen in oxidative extreme habitats and oxidize elemental hydrogen with CO_2, producing water and energy.
- Auto radio graphically has proven that methanogen incorporate carbon from CO_2 into complex organic matter.
- The biochemistry of this chemosynthesis is distinct from that involving RUBISCO, including photosynthesis.
- The metabolic portfolio of Archaea as a group also includes sulfur and metal oxidation.

In fact, phylogenetically diversity of Dissimilatory iron reducing bacteria (DIRB) belongs to Δ subdivision of the proteobacteria also exhibits phylogenetic relatedness to other gamma proteobacteria, lying in between archaea and eubacterial groups (Lundergan et al., 1996). Iron is present in minerals such as Ferrihydrite, Lepidocrocite, Vegemite, Magnetite, Hematite and Goethite. DIRB are mostly found in facultative to anaerobic environment of depth of ocean bottom, sediment of Lakes, Ponds and polluted coastal waters. Other bacteria competing among functional groups of anaerobic bacteria including nitrate reducers, Fe (III) reducers, sulfate reducers and methanogens for electron donors in rice paddy soil (Achtnichet et al., 1995). The DIRB occurrence was predicted as reduction of various electron acceptors have shown in addition of nitrate caused little or no inhibition of 0.5N HCl extractable Fe (II) but strongly inhibited sulfate reduction in paddy soil. This nitrate inhibition of iron is completely reduced as electron donors H_2 or fatty acids have added. Consequently, Fe (III) as amorphous oxide addition is caused partial inhibition of methanogens with concomitant decrease in the partial pressure of H_2 (Caccavo, Blakemore and Lovley, 1993A). *Bacillus infernus* species are grown in-vitro using substrates formate or lactate as the electron donor and Fe (III), Mn (IV), trimethylamine oxide or nitrate as the electron acceptor, (Boone et al., 1995). Enrichment culture studies suggests DIRB reduce only amorphous and crystalline iron oxides whereas Geothite and Hematite are relatively recalcitrant (Phillips et al., 1993). Moreover, iron reducers are also chelating agents in plants species and form siderospores and allows young plants to grow in soil strata.

The Fe (III) dissimilatory reduction associated with low grade (semi-taconite) iron ore (D'Christina, 1994). *Shewanella* alga strain BrY can gain energy for growth by reducing Fe (III) up to 30% of total Fe (III) in crystalline oxide goethite and hematite. Environmental parameters Fe (II) sorption to mineral and cell surfaces, surface area, pH and nutrients could grow coupling the oxidation of formate or lactate to the reduction of Magnetite (Fe(III) at pH 5-6, time 46 hrs. at temperature 22ºC (Kosice and Nealson ,1995). Wide variety of iron oxide FeO, Fe_2O_3 and iron bearing clay minerals constitute a substantial oxidation capacity in anoxic environment can serve as a source of Fe (III). DIRB was isolated from extreme and deep (Ca. 2700m) Triassic basin in Virginia. Number of iron reducing bacteria genera belong to *Desulfuromura* spp. Such as *D. kysingii*, *D. bakaii*, and *Desulfuromusa succinoxidans* capable of growing with Fe (III) as electron acceptor and phylogenetically related to the *Geobacter*/*Desulfuromonas*/*Pelobacter* groups belong to Geobacteriaceae (D'Christina and Dekong, 1993). This group have shown similarities with *Shewanella putrefacieans* (cyanobacteria) to sulfur reducer (table-1). Likewise Iron oxidizing bacteria live in acidic, aerobic environment rich in both reduced iron and sulfur compounds, is oxidize Fe(II) in presence of oxygen to (Fe(III) e.g. *Thiobacillus ferrooxidans*, *Thiobacillus denitrificans*.

TABLE 1

Bacterial occurrence, survival and utilization of different inorganic and organic energy sources in different habitat (Buchanan, R.E. & Gibbons, N.R, 1974 eds.) (Roberts Breed, E.G.D. Murray, Nathan R. Smith, (1957)

Sr. No.	Microorganisms	Presence & Isolated from Source	Morphology and Functional Role
1	Dissimilatory Iron Reducing Bacteria (DIRB), have shown similarities with *Shewanella putrefaciens* (Alga cyanobacteria) to sulfur reducer to ruman (gamma subdivision of the proteobacter) inhabiting *Bdellovibrio bacterivorous*	Dissimilatory Iron Reducing Bacteria (DIRB), isolated from marsh sediments sampled near Woods Hole Massachusetts USA;	Mostly found in facultative to anaerobic environment of depth of ocean bottom sediment of lakes, Ponds and polluted coastal waters. Geobacter lineage group /*Desulfuromusa*/*Peliobacter*/ *Geobacter hydrogenephilus* (Facultative Fe(III) reducing bacterium) *Desulfuromusa* spp. *D. kysingii, D. bakaii,* and *D.succinoxidans*
2	Geovibrio ferrireducens/ Geothrix fermentous is another novel DIRB that isolated Petroleum contaminated aquifer and phylogenetically distinc: closest relative of *G.fermentens* is *Holophaga toerida* a novel line of descent among bacteria	A phylogenetically distinct DIRB) isolated from hydrocarbon contaminated surface	Sediments and related to *Flexistripes sinusarabicil* (organisum not Fe(III) reducer); Bacterial strain *Wolinella succinogenes* phylogenetically related to isolate referred as SES-3 which grow with selenate as an electron acceptor
3	*Shewanella putrefaciens* group	Present in moderate saline environments	16S rRNA differentiating *S. alga* and *S. putrefaciens* and closely related to *Ferrimonas baltarica*,that exhibits phylogenetic relatedness to other Gamma proteobacteria
4	Spirillaceae: Vibrio sp., Desulfovibrio sp., Methanobacterium, cell vibrio, Microcyclus sp. Spirillum sp.,	Not known to use cellulose, Cellulose user	Cell simple, curved (spirillaceae) transverse division to form chains of spirally twisted cells; Slightly curved, non-motile, Anaerobic, *Desulfovibrio* sp. - Not known to attack cellulose; Slightly curved; non motile; *Methanobacterium* sp. -anaerobic; United in bundle; *Vibrio* sp.-cellulose attacked; *Microcyclus* sp.- Small slightly curved non motile rods; which form closed ring during growth; *Spirillum* sp.-cells form either screws or portions of a turn. Volutin granules are usually motile by means of a tuft of polar flagella
5	*Thiobacillus* sp.(Thiorhodaceae)	Iron deposits, Ocean sediments	Contain sulfur globule in presence of H2S, Oxidize sulfur compounds
6	*Gallionella* sp., *Siderophacus nausksia*	Iron rich sites	Young cells motile by single polar flagella, stalks secreted by the cells are slender and twisted
7	Sideromonas sp.	Iron and Manganese sites,	Cells spherical to ellipsoidal or bacilliform embedded in a thick mucilaginous capsule
8	Naumanniella	Iron rich sites	Cells ellipsoidal or rod shaped with rounded end occurring singly or in short chain
9	Ochrobium sp., Siderococcus sp., Ferrobacillus sp.- optimum reaction pH-3.5	Heavily impregnated with iron	Cells ellipsoidal or rod shaped cells that are partially surrounded by a marginal thickening
10	Salenomonas sp.,	Selenium sites	Cells kidney to cresent shaped with blunt ends
11	Clonothrix sp.	Iron and Manganese encrusted	Attached trichome showing false branching as sphaerotilus
12	Phragmidothrix sp.,	Iron deposits trichome are articulated unbranched and attached	The free ends being swollen surrounding the trichome are very thin, delicate
13	Crenothrix sp.	Iron and manganese oxide	Attached trichomes which are swollen at the free end
14	Peloplococeace: Peloploca sp., Pelonema sp.,	Iron rich sites	Cells 8 to 12 Microns long: trichomes are straight and a length up to 300µm
15	Bacterial genera of pond water *Aquaspirillum* sp. species *A.fasciculus* *Azomonas agilis* : Nitrogen fixer *Azorhizobium caulinodans* *Azotobacter chroococcum* *Beiherinckia indica* *Bordetella pertussis*	pond water	Transformation of Organic matters Respiratory type of metabolism and can use oxygen as terminal electron acceptor. Nitrogen fixer some genera can respire anaerobically with nitrate, fumarate or other terminal electron acceptors.

Sr. No.	Microorganisms	Presence & Isolated from Source	Morphology and Functional Role
	Bradyrhizobium japonicum (3-ketoglycosides are not produced) *Brucella melitensis* *Chromohalobacter marismortui* *Chryseomonas lutcola* *Comamonas terrigena* *Capriavidus accator* *Deleya aquamarina* *Deoxia germnosa* *Ensifer adhaerens*		
16	**Gram negative Aerobic / Micro aerophilic Rods and Cocci** *Acetobacter aceti* *Acidiphilium cryptum* *Acidomonas methanotia* *Acidothermus cellulolyticus* *Acidovorax facilis* *Acinetobacter calcoaceticus* *Afipia felis (BCYE)* *Agribacterium fumefacieus* *Agromonas oligotrophica* *Alcaligenes faecalis* *Alteomonas macleodi* Denitrification seawater base for growth *Aminobacter aminovorans* –PYG Broth		Respiratory type of metabolism and can use oxygen as terminal electron acceptor. Nitrogen fixer some genera can respire anaerobically with nitrate, fumarate or other terminal electron acceptors.
17	Azotomonas sp.	Soil and free living	Indole producer, Free nitrogen aerobic
18	Eubacteriales: Azotobacteriaceae, Azotobacter chroococcum	Agri. soil habitat	Gram negative, rod shape-Aerobic or Facultative anaerobic-Large ovoid to rod shaped cell, free living on soil, free nitrogen
19	Rhizobiaceae	Heterotrophic rods which may not require organic nitrogen for growth usually motile by means of one to six flagella. Frequently form nodules or tubercles or roots of plants	or show violet chromogensis colonies usually large and slimy, especially on mannitol agar

Modern Techniques

Laboratory methods for recovery enhancement of stressed organisms: Some of precautionary measures such as samples containing heavy metals (after samples making dilution) were inoculated test medium within 30 minutes (i.e. enriched non inhibitory medium at moderate temperature). When multiple-tube fermentation test result in consistently higher counts than those obtained from membrane-filter tests, in such cases sample contain stressors such as disinfectants or heavy metals resulting in injured bacteria. Recovery of injured bacteria & suboptimal recovery based on one or more of the following procedure as described follows. Before application of cultural methods, field sample shell be processed in research laboratory under following guidelines Collection of water samples with elevated concentration of heavy metals ions is nullified in sampling bottles containing a chelating agent e.g. Sodium salt of EDTA and minimize sampling storage time (APHA, 2005). Secondly use of buffered peptone dilution water rather than buffered K_2HPO_4 water (section 9212 B). As we know water from municipal corporation water supply, are chlorinated in treatment process before city water distribution system. Bacterial samples higher recovery is possible by adding sufficient dechlorinating agent 10% solution of sodium thiosulfate 0.5 ml added to sampling bottle before Autoclave sterilization of sampling bottles (section 9060 A).

Bacterial counting: The inoculated plates with water samples shall be incubated at specified temperature and time. Bacterial counts /ml or /100ml shall be expressed as to transform count in to log transformed result. For two lot of plates presented in parallel column, the calculated the difference (**d**), between the two transformed result for each sample as the bacterial count mean (**x**) and standard deviation (**SD**),

TABLE 2
Few bacterial groups and subdivision characteristics (Woese, C.R., 1987)

α- subdivision: Purple non sulfur bacteria	Rhizobacteria, Agrobacteria, Rickettsia, Nitrobacter
Β- subdivision	Rhodocyclus, (some) Thiobacillus, Alkaligenes, Spirillum, Nirosovibrio,
Γ-subdivision	Enteric, florescent pseudomonads, purple sulfur bacteria, legionella, (some) Beggiotoa
Δ- subdivision	Sulfur and sulfate reducers, (Desulfovibrio), Myxobacteria, Bedellovibrios
Gram positive eubacteria A. High G+C species	Actinomyces, Streptomyces, Arthrobacter, Micrococcus, Bifidobacterium
B. Low G+C species	Clostridium, Peptococcus, Bacillus, Mycoplasmas
C. Photosynthetic Species	Heliobacterium
D. Species with Gram negative walls	Megasphaera, Sporomusa
Cyanobacteria and Chloroplasts	Aphanocapsa, Oscillatoria, Nostoc, Synechococcus, Gleobacter, Prochloron
Spirochetes and relatives	A. Spirochetes : Spirochaeta, Treponema, Borrelia B. Leptospiras: Leptospira, Leptonema
Green sulfur bacteria	Chlorobium, Chloroherpetus
Bacterioids, Flavobacteria and relative	A. Bacteroides, Fusobacterium B. Flavobacterium group-Flavobacteriu Cytophaga, Sporospira Flexibacter
Planctomyces and relatives	A. Planctomyces group Planktomyces, Pasteuria B. Thermophile Isocystis pallida
Chlamydiae	Chlamydia psittaei: Radio resistant micrococci and relatives A. Deinococcus group -Deinococcus radiodurans B. Thermophiles Thermus aquaticus
Green non sulfur bacteria relatives	A. Chloroflexus group-Chlroflexus sp., Herpetosiphon B. Thermomicrobium group- Thermomicrobium rosentu

How far molecular studies will confirm the taxa recognized at present? And how soon it will be possible to use molecular approaches routinely? As a rapid means of characterizing cells using library source from natural populations. Unfortunately, the various "phylogenetic tree" illustrating similarities between strains based on similarities between particular parts of the genome (16S-rRNA or whole genome sequences) i.e. researches in late 1990 and early 2000s often used for comparisons from strains whose generic and specific names are doubtful. This restricts the value of such phylogenetic trees for comparing possible evolutionary relationships with the taxonomic relationships suggested by classical names, i.e. general theory for the evolutionary relationships among the main bacterial phyla (Paquin et al., 1997).

The principles and materials and methods, applications involve in these techniques are briefly discussed. Methods for determining the Viable but not Cultivable state (VBNC State) (Salle, 1973)

1. Direct Viable Count using cellular integrity and staining of nucleic acid with Acridine Orange or 4'-6' Diamidino -2-Phenyl Indole (DAPI).
2. Detection of Respiration by (i) (P-iodo-nitro-tetrazolium violet) an electron acceptor (ii) 5-Cyano-2,3,ditolyl -tetrazolium chloride (CTC)
3. Monoclonal antibodies (Use of Fluorescent labeled monoclonal antibodies (MAbs)
4. Loss of radio-labeled (3H) Thymidine
5. Intracellular ATP levels
6. Flow Cytometry

These techniques were advanced later on using below mentioned techniques (Jeffrey et al., 1991) such as Epifluorimetry-Image analysis. Fluorescence microscopy can also be used based on specific colour taken up by internal organelles to detect the viable bacterial cells out of non-cultivable viable state of Bacteria (NCVB). Gene probes/Polymerase chain reaction.i.e. Identification species of bacteria by polymerase chain reaction (PCR), Monoclonal antibodies, Biosensors (chemical sensors based detection strategies), and Laser cytometry.

Epifluorimetry - Image analysis: Conventional methods are used in the laboratory to detect and quantify bacteria are plate count colony forming unit (CFU). Plate culturing is the "Gold standard" for bacteria detection. Fluorescent dyes such as propidium dye and SYTO-9 can be used for this purpose. While propidium iodide is membrane impermeable, SYTO-9 can permeate the membranes and thus identify all the bacterial population (https://www.news.medical.net). Cellular fluorescence provides a sensitive index of the functional state of a living cell and hence may be used to determine their metabolic status.

Fluorescence Spectroscopy Technique: Fluorescence spectroscopy provides detailed information about the electronic states of a molecule (e.g. ATP to cAMP, energy transfer, relaxation, intersystem crossing) is common structure to all organisms from bacteria to higher organisms (Maruyama, 1991). Useful in academic research and real world application some molecule when they are hit by a photon they can absorb the energy of that photon to get into excited state. Detail information about the electronic states of a detailed information about the electronic states of a molecule (e.g. energy, polarity) and about the processes that takes place after light absorption (e.g. energy transfer, relaxation, intersystem crossing as well as

their time scales. Method is relevant for taking & selecting materials for optoelectronic devices such as LEDs and Solar cells. Application can be broadly split into two main groups distinguishing life science, with chemicals and materials (including academia). The second has seen huge growth in the use of fluorescence instruments for use in cell analysis. Some molecules are naturally fluorescent. Such as

- Proteins and peptides (tyrosine, tryptophan, phenylalanine)
- Nicotinamide adenine dinucleotide (NAD)
- Riboflavin
- Chlorophyll
- Quantum dots
- Quinine
- Minerals such as Calcite, Apatite, Corundum
- Fluorescence dyes such as Rhodamine, BODIPY, Fluorescein
- (Ref. - https: www. Agilent.com, Ethidium bromide stains in whole blood sample- (https://www.ncbi.nih.gov.in; https://www.ionoptix.com.)
- The reaction uses fluorescent reactant and/or products i.e. fiber-optic probe for measurement directly in a reaction flask and extremely rapid scan rates for the analysis of short lived species. The reaction diminishes slowly dependent on reactants.
- Fluorescence spectroscopy is versatile method for their ability to perform kinetic measurements of Life science applications.
- Detect intracellular ion concentrations and determination of intracellular analytes concentrations.
- Read - out of fluorescence - based immunoassays.
- Analysis the rotational motion of molecules using polarizing filters under finely control sample temperature (Determining the thermal stability of biocatalysts and pharmaceuticals application includes: Determining the melting temperature ™ of DNA and RNA samples).
  - Characterizing bio labels for live cell imaging.
  - Characterizing G-protein coupled receptors (GPCR) oligomerization.
  - Detecting specific bacterial strains using fluorescent assays luminescent bacteria *Photobacterium* spp. & *Vibrio* spp. (Ranran Du et al., 2022).
  - Understanding platelet response using cellular signaling and identifying aggregated biomolecules such as monoclonal antibodies.
  - Analyzing changes in the tertiary structure of proteins.

Image Analysis

The technique is key driver of identify the morphological features in biomedical science i.e. interaction of light with biological sample viz. compound microscopy, phase contrast microscopy, confocal microscopy, fluorescence microscopy and non - linear optical microscopy which may be limited by quality of out-put image, post image processing or instrumentation cost, (Lecun et al., 2015).

This automatic image analysis has advantage over manual image analysis as fast and robust image analysis which machine algorithm designed to function as an artificial human brain. In this you have to design ANN, and compiling layers of interconnected nodes or "neurons" that process and delivers well processed image with defined image quality criteria, as desired using tools like machine learning (ML) algorithms such as support vector machine (SVM) and artificial neural network (ANN) (Rivenson et al., (2017). Cluster analysis are commonly implemented in the methods which ANN have proved to be effective in addressing classification problems. The ANN comprised a fundamental unit known as perception (Hornik et al., 1989). Functional part of biological neural network (Perception) consists of ANN input layer, a hidden layer, and an output layer, (Fig.-1 to 3). The hidden layers may have a variable number of perceptions to be optimized. Overall input to every layer is drawn up to the output of the preceding layer which is processed and translated to the next layer i.e. activation function (Melanthota et al., 2022). The final number of data points present in the dataset of the image will produced and analyzed.

Gene Probes/Polymerase Chain Reaction: The method is used for detection of low levels of microorganisms in environmental samples. In this method, the bacterial samples for DNA extraction was done adding 100µl diethyl pyro carbonate (sigma) autoclave sterilized was added to a set of tubes containing bacteriological samples. Earlier bacterial cells collected by using fluoropore filters (Millipore Corp.), then filters were added in diethyl pyro carbonate containing tubes. The sample test tubes were subjected to six cycles of freeze-thaw lysis. The gentle release of total genomic DNA from bacterial cells has been preserved in presence of lysozyme. The hydrophobic Durapore HVHP and CVHP filter (Less affinity to proteins) Polyvinylidene fluoride (PVDF) Teflon (Millipore) and from several other hybridization nylon and nitrocellulose membranes sources were also treated 1x to 10x Denhardt's solution at 45ºC for 1 h with gentle shaking washed with Tris - EDTA buffer (pH 8.0) Gross man and Ron, (1975) and Ron et al., (1966), and used for the filtration of the sample containing bacterial cells. (100X Denhardt's solution contained 2% ficoll (molecular weight 400,000), 2% polyvinyl pyrrolidone molecular weight 360,000) and 2% bovine serum albumin (nuclease free) (3), before and after freeze thaw cycle, three of the aliquots were separated (1) one of the aliquots was plated on nutrient –agar and grown for 48h at 37ºC to determine viable CFU. (It is further subject to compare post lysis bacterial cell determination of the original samples microbial plate count). (ii) The second aliquot was used for acridine-orange direct count (9) determination of total cell number. (iii) The third aliquot was used for PCR amplification, initial cell number in the culture was determined from the number of colonies which appeared on the plate prior to freeze-Thaw cycles.

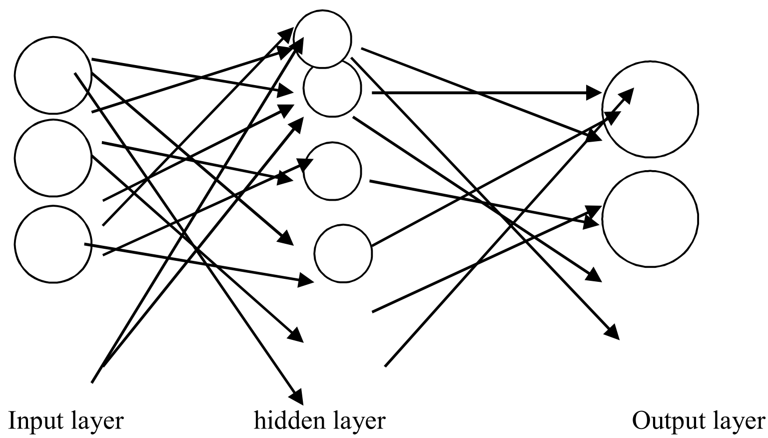

Figure-1: ANN of artificial interconnected nodes for input layer, a hidden layer, and an output layer

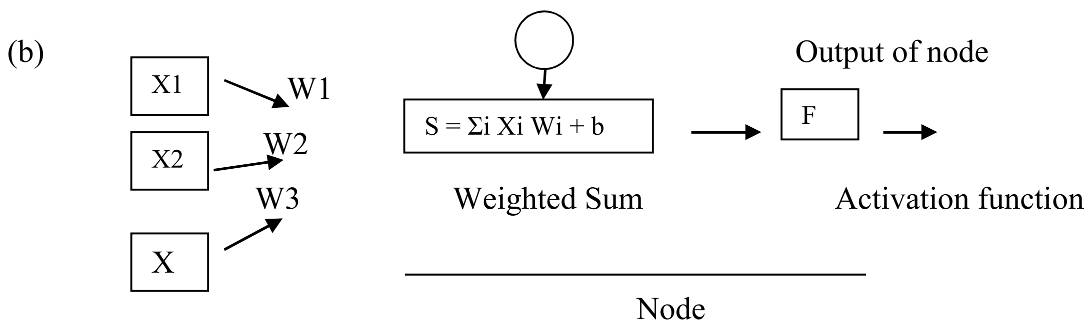

Figure-2: Neural network for enhancing 3 D optical microscopy images.

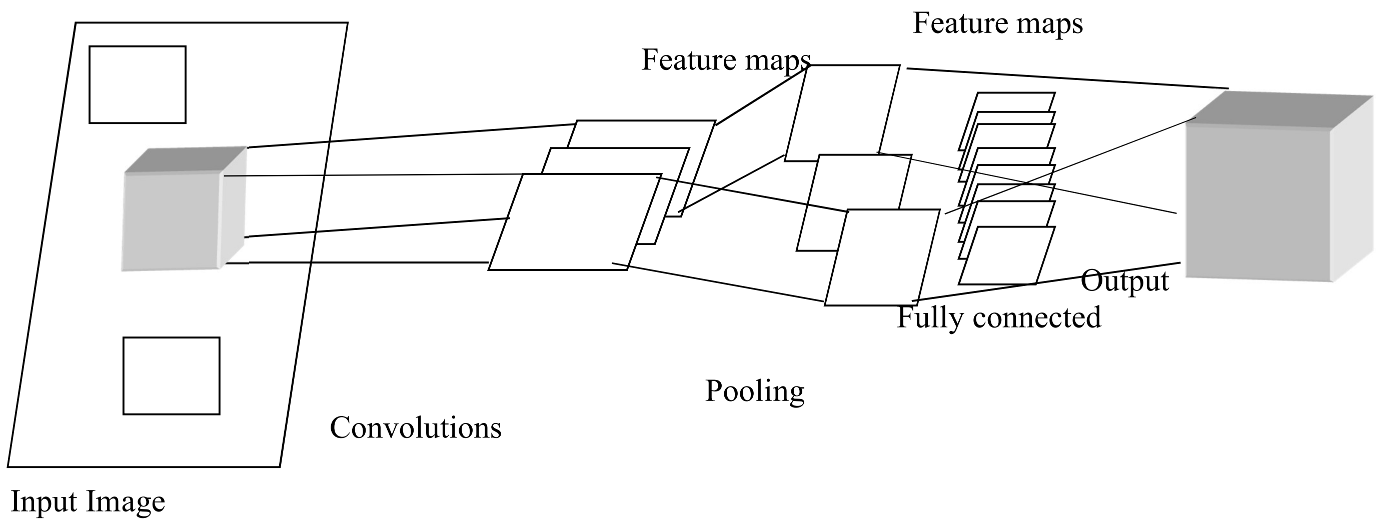

Figure-3: Artificial neural network (ANN)

The Teflon fluoropore hydrophobic, poly tetra-fluoro-ethylene-polymer membrane (PTFE – (Millipore Corp.) were soaked in ethanol. Hydrophilic filters FHLP including other were soaked in sterile water until they become transparent (<1min) (FHLP and FGLP). Each filter was then transferred to a swinnex filter holder (Millipore) for filtration. The shiny surfaces were used for collecting the bacterial cells on hydrophilic filter fluoropore FHLP0013 and 0025 and FGLP0013 and 0025 filters. In cell detection process each sample was frozen in ethanol-dry ice bath for 1 min and then thawed by transferring it to a 50ºC water bath for 1 min without lysozyme. The samples were vortexed vigorously for 10 to 15s after every two cycles of freeze-thawing to release the cells or nucleic acids from the filter surface. Percent cell lysis was calculated from the initial number of colonies which appeared on the plate prior to freeze-thaw cycles. The samples were then transferred to a DNA thermal Cycler (Perkin-Elmer Cetus) and heated to 85ºC for 3 to 5 min to inactivate proteases and nucleases to protect the AmpliTaq DNA polymerase enzyme and primer or target nucleic acids from possible destruction or damage. The samples were then cooled to room temperature and spun for 2 to 3s in an Eppendorf micro centrifuge to collect any condensation from heating the samples.

The PCR mix was added to each sample. Primer-directed amplification of the target DNA was performed without further purification.

PCR Mix: A total volume of 150µl of PCR mix containing 5µl of 10C reaction buffer (500mM Tris-Cl (pH-8.9) 500mM KCL, 25mM [in same cases up to 80mM $MgCl_2$], 24µl of deoxy-nucleotide triphosphate (dNTP) mix (final concentration of 200µl each dNTP, Perkin –Elmer Cetus), 0.2 to 1.0 µl each primer and 5U (1µl) of amphitag DNA polymerase (Perkin-Elmer Cetus) added in each tube. The samples were spun again for 2 to 3s to collect any liquid on the wall or on the cap of the tube. the of the filter was folded with a sterile needle and submerged in to the aqueous phase to prevent contact between the mineral oil and the filter, before adding the mineral oil. Sterile mineral oil (80µl) (Sigma) was added to the top of the sample.

Triple PCR amplification was performed with primers for the Lac Z, Lam B and Uid R genes for *E. coli*. In triplex PCR amplification typically 1ng of purified genomic DNA of E. coli was amplified with equimolar quantities (0.5µm) of each of the three sets of Lac Z, Lam B, and Uid R primers at annealing temperature of 60ºC for a total 30cycles

i. For detection of total coliform bacteria, a 264bp region located closer to the amino-terminal end of the LacZ gene of E. coli was amplified by using two 24 - mer primers, (Bej et al., 1991), (LZL-389, LZR-653). A 366bp segment of the coding region of the LamB gene of E. coli was amplified by using two 24-mer primers (LBL-445, LBR-790).
ii. For more specific detection of E. coli and other enteric pathogens such as *E. coli* and *Shigella* species 154bp of the regulatory region of Uid A gene designated, Uid R which is located upstream of the Uid A structural gene were amplified by using the 22'mer primers URL-301, URR-432). In triplex PCR amplification, typically 1 ng of purified genomic DNA of E. coli was amplified with equimolar quantities (0.5µm) of each of the three sets of Lac Z, Lam B, and Uid R primer at annealing temperature of 60ºC for a total of 30 cycles.
iii. For the detection of genus *Legionella* and /or *L. pneumophila* a duplex amplification with L mipL 920 and L mipR 1548 for the 5S rRNA gene as targets was performed. A 650bp macrophage infectivity potential or mip gene of *L. pneumophila* was amplified with two 21-mer primers Lmip L920 and Lmip R 1548 for detection of serogroups of *L. pneumophila*.
iv. The mip and 5S rRNA primer sets were used in a ratio of 5:1 respectively, when duplex amplification were performed. For detection of the total genus of Legionella, a 104bp region of the 5S rRNA gene was amplified by using two 20-mer primers. L5SR9 and L5SR93.
v. To demonstrate method two to three bacterial culture and their oligonucleotide probes were radiolabeled at their 3' OH ends with [α^{32} P] dCTP [Cytosine triphosphate] (Specific activity, 3000Ci/mmol) (Dupont, New Research Products Boston, Mass.) By terminal deoxyribo-nucleotidyl transferase (IBI) or at their 5' ends with [γ^{32}P] ATP [Adenine triphosphate] (specific activity >3,000 Ci/mmol) Ns) New Research Product by using polynucleotide kinase (U.S. Biochemical Corp.)
vi. Following the procedure described by Ausubel et al., (1989) as these genome sequences of 200 to 300ng of respective radio labeled oligonucleotide probe from gene library and specific activity of each probe was $5x10^7$ to $1x10^9$ cpu/µg of DNA was used as determined by Beckman LS5000TD Scintillation counter. For each hybridization 200 to 300ng of the respective radiolabeled oligonucleotide probe was used.
vii. PCR amplified DNA were detected by using gel electrophoresis and radiolabeled gene probes. The DNAs were separated by using a 0.8 to 1% horizontal agarose gel, a 4% (Nusieve-Seakem, 3:1) (FMC Bioproducts Rockland Maine) agarose gel with Tris-acetate-EDTA buffer or a 10% vertical Polyacrylamide gel with Tris borate-EDTA buffer.
viii. The gels were stained in 2X10-4 % ethidium bromide solution, visualize with a photo/prep 11UV trans-illuminator (Fotodyne, Inc., New Berlin W.S.) and photographed.

For Southern and dot blot hybridization, the DNAs were denatured and transferred on to Zeta probe nylon membranes (Bio-Rad Laboratories, Richmond Calif.) by following the procedure described by the manufacturer. All hybridizations were performed at 55 to 60ºC for 16h, using hybridization solution described bt manufacturer, (Bio-Rad). The blots were washed twice in 2 x SSPE (1 X SSPE is 0.18M NaCl, 10mM $NaPO_4$, and 1 mM EDTA (pH 7.7) 0.5% SDS at 45ºC for 10min each and once in 0.1 x SSPE -0.1% SDS at 53ºC for 3 to 5 min with gentle agitation. To detect 32P – radio labeled DNAs, the blots were covered with Saran wrap (Fisher Biochemical Pitts burg, Pa.) and X-ray film (X-AR film) Eastman Kodak Co.

Rochester, N.Y.) was placed over them, Film exposed was at -70ºC for 1 to 16h.

Monoclonal antibodies: In Immuno-histochemistry, monoclonal antibodies can be used to detect antigens in fixed tissue sections, and similarly, immunofluorescence can be used to detect a substance in either frozen tissue section or live cells.

The functional characteristics of monoclonal antibodies:

- The monoclonal antibodies being directed against single epitopes are homogeneous, highly specific and can be produced in unlimited quantities. In animal disease diagnosis, they are very useful for identification and antigenic characterization of pathogens, (Reference: Conway & Macario, ASM news) monoclonal antibodies for bacterial identification and taxonomy.
- This worrisome trend warrants the development of Non-antimicrobial approach is to combating infection caused by these isolates. In addition to the paucity of new antibiotics with novel mechanism of action in the development in pipeline. Monoclonal antibodies (mAbs) (B cell of Lymphocytes produce monoclonal Abs) have emerged as highly effective molecules for the treatment of multiple diseases.
- However, in spite of the fact that antibodies of lymphocytes play an important role in protective immunity against bacteria, only three mAbs therapies have been approved for clinical use in the bacterial infections i.e. Raxibacumab, Obiltoxaximab, and Bezlotoxymab.
- The therapeutic potential of mAbs in the treatment of bacterial diseases and discuss how their development can be facilitated when assisted by "Omics" technologies and interpreted under a systems biology paradigm. Specifically method employing genomics, transcriptomic structural and proteomic data sets allow for the rational identification of epitope's highly conserved at the amino acid level, surface exposed, located on antigens essential for virulence, and expressed during critical stages of infection.

Therefore, these knowledge base approaches can contribute to the identification of high-value epitopes for the development of defective mAbs against challenging bacterial clones, (Antonio et al., 2019).

Biosensors: Fluorescence based dyes that can discriminate bacteria (Huang et al., 2021, Yoon et al., 2021) For example PCR methods use amplified nucleic acids to identified DNA sequences is time consuming expensive require complex procedures. However, Immunological techniques utilize the antibody and antigen and Raman spectroscopy can distinguish the bacteria based on difference of light scattering.

Applications:

- Nuclear vs. cytoplasmic localization of fluorescence
- The micronucleus assay and DNA damage signaling
- FISH Analysis – Fluorescence in-situ hybridization cytogenetic studies
- Analysis of Nucleoli and Protein translocations between Nucleoli and Nucleoplasm
- Progeny of individual cells/Clonogenicity assay
- Cell Immuno-phenotyping, visual cell examination; Image enzyme kinetics and other time resolved events; LSC in Clinical Pathology Utility of LSC in other application

Propidium iodide (or PI) is a fluorescent intercalating agent that can be used to stain cells and nucleic acids. PI binds to DNA by intercalating between the bases with little or no sequence preference. When in an aqueous solution PI has a fluorescent excitation maximum of 493nm (blue green) and an emission maximum of 636nm (red) after binding DNA; the quantum yield of PI is enhanced 20-30 fold and the excitation/ emission maxima of PI is shifted to 535nm (green)/617nm (orange-red).

Propidium iodide is used as DNA stain in flow-cytometry to evaluate cell viability or DNA content in cell cycle analysis or in microscopy to visualize the nucleases and other DNA-containing organelles. Propidium iodide is not membrane permeable, making it useful to differentiate necrotic apoptotic and healthy cells based on membrane integrity. PI also bind to RNA necessitating treatment with nucleases to distinguish between RNA and DNA staining (s). PI is widely used in fluorescence staining and visualization of the plant cell wall, (https://en.wikipedia.org/wiki/propidium_iodide).

SYTOX (also known as SYTOX Green) stain name- SYTO 9: SYTOX is a high affinity nucleic acid stain developed by biotechnology company molecular probes. Because the stain only penetrates cells with compromised plasma membranes. It can be used to investigate antibacterial mechanism of action and confirm loss of bacterial viability. There have however, been studied which confirm the use of SYTOX dyes for live cell imaging of bacteria, (https://en.wikipedia.org/wiki/SYTOX).

SYTO 9 stain is particularly useful as a nuclear counter stain for bacterial assays. Since it stains both live and dead Gram +ve and Gram –ve bacteria. SYTO 9 is a green fluorescent nuclear and chromosome counterstain that permeate to both prokaryote and eukaryotic cell membranes. It has high affinity for DNA and exhibits enhanced fluoresce upon binding with an excitation maximum at 483nm and fluoresce emission maximum at 503nm. SYTO 9 is used in flow cytometry and fluorescence microscopy.

Laser Scanning Cytometry:

Method:

The microscope identifies the center of initiating observations of bacteria or biological sample as object which provide essential structural and optical components (Pozarowski et. al., 2013).

The fluorescence excitation layer beams from up to four lasers, spatially merged by dichromatic mirrors are directed in to the computer controlled oscillating (350Hz) mirror which reflects them through the pea-illumination port of the

microscope and images through the objective on to the slide. The mirror oscillates and laser beams to sweep the area of microscope slide under the lens. The spot size varies depending on the lens magnification from 2.5µm (at X40) to 10µm (at X10). The slide was scan on its xy position 0.5µm steps per each laser scan on computer-controlled motorized microscopic stage monitored by sensors & Light intensity was recorded by sensors.

The specimen emitted fluorescence is collected by the objective lens and directed to the scanning mirror. Upon reflection, it passes through a series of dichromatic mirrors and optical emission filters to each one of the four photomultipliers. The recorded image fluorescence at a specific wavelength, defined by the combinations of filters and dichromatic mirrors.

A light source additional to the lasers provided transmitted illuminations to visualize the objects through an eye piece on the charge coupling device (CCD) camera.

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